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Sleep consolidates motor learning of complex movement sequences in mice

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Conflicts of interest

G. Tononi is involved in a research study in humans supported by Philips Respireonics. This study is not related to the work presented in the current manuscript. The other authors have indicated no financial conflicts of interest.

33 **Abstract**

34

35 **Study Objectives**

36 Sleep-dependent consolidation of motor learning has been extensively studied in humans, but it
37 remains unclear why some, but not all learned skills benefit from sleep.

38

39 **Methods**

40 Here we compared 2 different motor tasks, both requiring the mice to run on an accelerating device. In
41 the rotarod task mice learn to maintain balance while running on a small rod, while in the complex
42 wheel task mice run on an accelerating wheel with an irregular rung pattern.

43

44 **Results**

45 In the rotarod task, performance improved to the same extent after sleep or after sleep deprivation.
46 Overall, using 7 different experimental protocols (41 sleep deprived mice, 26 sleeping controls), we
47 found large interindividual differences in the learning and consolidation of the rotarod task, but sleep
48 before/after training did not account for this variability. By contrast, using the complex wheel, we
49 found that sleep after training, relative to sleep deprivation, led to better performance from the
50 beginning of the retest session, and longer sleep was correlated with greater subsequent performance.
51 As in humans, the effects of sleep showed large interindividual variability and varied between fast and
52 slow learners, with sleep favoring the preservation of learned skills in fast learners and leading to a net
53 offline gain in performance in slow learners. Using Fos expression as a proxy for neuronal activation,
54 we also found that complex wheel training engaged motor cortex and hippocampus more than the
55 rotarod training.

56

57 **Conclusions**

58 Sleep specifically consolidates a motor skill that requires complex movement sequences and strongly
59 engages both motor cortex and hippocampus.

60

61

62 Key words: sleep-dependent consolidation, motor learning, sleep deprivation, rotarod, complex wheel

63

64 **Statement of Significance**

65

66 Sleep benefits some types of memory and not others, but the reasons why remain unclear. We
67 employed 2 different motor tasks, the rotarod task and a novel complex wheel task, and found that
68 sleep specifically consolidated motor learning exclusively in the latter. In both tasks mice run on an
69 accelerating device but only the wheel task requires acquisition of complex movements with high
70 spatial accuracy. Immunocytochemical analysis of Fos expression revealed that compared to the
71 rotarod task, the complex wheel task induces higher neuronal activity in motor cortex and
72 hippocampus but comparable activity in other areas including medial prefrontal cortex and striatum.
73 Thus, sleep specifically consolidates motor learning with complex movement sequences.

74

75

76 **Introduction**

77 The beneficial effects of sleep in motor learning ¹⁻⁶ are well established in humans, and the evidence is
78 compelling for motor sequence learning, in which subjects are asked to perform complex movement
79 sequences as quickly and as accurately as possible. Specifically, numerous studies of sequence
80 learning that used finger-tapping, finger-to-thumb opposition and other paradigms ⁷ reported that
81 nighttime sleep as well as a post-training daytime nap favored consolidation of motor skills and
82 improved task performance in subsequent sessions ¹⁻⁶. Brain imaging studies have shed light on the
83 interaction between hippocampus, striatum and prefrontal cortex during learning and consolidation of
84 procedural memory ^{8,9}. However, the mechanisms underlying the sleep-dependent refinement of motor
85 skills are still poorly understood. Thus, the essential requisites that determine whether a learned skill
86 will benefit from sleep remain unclear and controversial ¹⁰⁻¹². For instance, on one hand there is
87 evidence that the explicitness of the sequence to be learned is critical for sleep-dependency ^{10,11}. On
88 the other hand, several other studies found beneficial effects of sleep in motor adaptation tasks, which
89 require implicit learning ¹³⁻¹⁵. There is also some evidence that more difficult tasks benefit more from
90 sleep, but this conclusion was reached by comparing tasks that were all sleep-dependent ¹⁶.

91 Sleep-dependent consolidation of motor skills is much less documented in animals. In the
92 rotarod task mice or rats learn to maintain their balance and run on a small rod that rotates at a constant
93 acceleration, and the speed when the animal falls off the rod is recorded as measure of performance ¹⁷⁻
94 ²³. Previous studies using one training session per day found that rotarod performance shows fast
95 improvement within a session and a slower improvement across sessions. Intrasession improvement
96 diminishes across days, and performance reaches a plateau within 3-5 days ^{19, 20, 23}. A recent study
97 compared the next day improvement in rotarod performance in mice that were either sleep deprived or
98 allowed to sleep after training ²². Both groups performed better the next day, but the improvement was
99 reduced approximately by half (from 44 to 23%) in the sleep deprived mice. However, that work could
100 not establish whether sleep promoted fast, intrasession learning and/or offline consolidation. Very few
101 other studies in rodents have used tasks that require the acquisition of complex movement sequences.
102 One is the reaching task, in which rodents learn to approach a small opening in the front of the
103 recording chamber, determine whether a sucrose pellet is available on the shelf and, if so, reach
104 through the opening to retrieve the pellet with the preferred paw ^{24, 25}. In rats, 2h of post-training sleep
105 led to faster reaching movements relative to 2h of sleep deprivation, with no decrements in accuracy ²⁴.
106 In mice instead, 5h of post-training sleep did not provide an immediate advantage over an equivalent

time of forced wake²⁵. Mice that could sleep did show a delayed gain in performance 24h after training, but improvement was measured across the entire session without teasing apart the offline consolidation from any additional learning during retest²⁵. In summary, the evidence that sleep benefits motor skill learning and/or sequence learning is scant in rodents. Yet, the characterization of sleep-dependent motor tasks in mice would pave the way to the use of genetic, molecular, and electrophysiological approaches to understand how sleep benefits learning and memory.

Here we aimed at clarifying whether in mice sleep promotes specific forms of motor learning and if so, whether it facilitates intrasession learning, offline consolidation, or both. We used 2 tasks, the rotarod task and a modified version of the “classical” complex wheel running task²⁶⁻³⁰, in which we trained mice to run on top of an accelerating wheel that lacks some rungs at random, rendering the rung pattern irregular and highly complex. Both tasks require the mice to run on an accelerating device and involve a short first training session (~1h) without pretraining or food restriction. However, compared to the rotarod task, the complex wheel task has an additional motor sequence learning component, as the acquisition of the exact position of the paws and the precise sequence of movements are required to run on the wheel. We find no evidence for sleep-dependent consolidation after rotarod training. By contrast, we show that the complex wheel task, which is more difficult than the rotarod task and leads to stronger activation of motor cortex and hippocampus, benefits from sleep. Thus, we provide, to the best of our knowledge, the first evidence of offline, sleep-dependent consolidation of sequence learning in mice and identify some of the factors that make a task sensitive to the effects of sleep.

Methods

Animals. B6.Cg-Tg(Thy1-YFP)16Jrs/J mice (YFP-H, Jackson Laboratory) were maintained on a 12 h light/12 h dark cycle (lights on at 8AM) with food and water available *ad libitum*. YFP-H mice express yellow fluorescent protein (YFP) in a subset of cortical pyramidal neurons³¹, and thus can be used to study the link between sleep and synaptic plasticity³²⁻³⁴. In total, we used 67 mice (52 males and 15 females) for behavioral experiments with the rotarod task, 188 mice (121 males and 67 females) for a complex wheel task, 4 mice (3 males, 1 female) for a regular wheel task and 15 additional male mice for Fos immunohistochemistry (4 sleeping controls, 3 mice for rotarod 20 trials, 4 for rotarod 40 trials and 4 for complex wheel 20 trials) (Table S1). In each experiment most, if not all, mice were litter-matched. All animal procedures and experimental protocols followed the

138 National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by
139 the licensing committee. Animal facilities were reviewed and approved by the institutional animal care
140 and use committee (IACUC) of the University of Wisconsin-Madison, and were inspected and
141 accredited by the association for assessment and accreditation of laboratory animal care (AAALAC).

142
143 **Sleep recordings and sleep deprivation.** Experiments were done in adolescent mice (P29-36, mostly
144 P29-32) (Table S1). It was previously shown that 1-month old YFP-H mice have consolidated
145 sleep/wake patterns and homeostatic sleep regulation similar to adult mice ³³. Sleep and wake states
146 were determined by continuous monitoring with infrared cameras (OptiView Technologies) starting
147 at least 24 h before the first training session. This method cannot distinguish NREM sleep from
148 REM sleep, but it consistently estimates total sleep time with $\geq 90\%$ accuracy ³². Motor activity was
149 quantified by custom-made video-based motion detection algorithms (Matlab), as previously
150 described ³⁵. Sleep deprivation (SD) was enforced using 2 methods: gentle handling, in which mice
151 were touched with a cotton swab, and exposure to novel objects, in which toys and other objects of
152 different shape, color and texture were introduced in the cage. In both cases mice were stimulated only
153 when they appeared drowsy, assumed a typical sleeping position, and/or closed their eyes. Mice were
154 never disturbed when they were spontaneously awake, feeding or drinking. During SD (7h), mice were
155 awake $95.0 \pm 0.36\%$ of the time (SD with gentle handling, SDgh) and $93.7 \pm 0.46\%$ of the time (SD
156 with novel objects, SDob). During the same 7h, mice allowed to sleep were awake $28.4 \pm 0.77\%$ of the
157 time.

158
159 **Rotarod.** Four individual accelerating rotarod systems (EZRod, Omnitech Electronics, Inc.) were used,
160 each system controlled separately. Prior to the first training, all mice were weighed. Mice were placed
161 onto a stationary rod and acceleration began. The acceleration profiles were fast (0 to 100 rpm in 3
162 min) or slow (0 to 80 rpm in 5 min), with the fast protocol used in most experiments, as summarized in
163 Table S2. The actual acceleration in SI units was 314 cm/min^2 and 150.7 cm/min^2 in fast and slow
164 protocol, respectively. Time and speed when mice fell off the rod were automatically recorded.
165 Sometime a mouse unable to keep up with the increasing speed would grab the rod to stay on it
166 without running. In these cases we gently pushed the animal off the rod, and we counted these trials as
167 well. Each training session included 20 or 40 consecutive trials. Every 10 trials mice were returned to
168 their home cage for a 5 min rest period, during which mice mainly groomed, but never slept. Since

backward running is more difficult than forward running, mice had to be forced to train in the second paradigm by using a home-made anti-flipping tool made of 2 parallel plastic boards with adjustable distance between them, which forced the mouse to maintain the backward direction (Fig. 1A). As in the previous study²², the acceleration profile of backward training was 0 to 50 rpm in 3 min.

Surgery

To mimic the experimental conditions of the previous rotarod study²², a subset of mice underwent surgery and was implanted with EEG electrodes. Mice were anesthetized with isoflurane (3-5% for induction, 1-2% for maintenance) and positioned in a Kopf stereotaxic apparatus. After the skull was exposed, two screw-type EEG electrodes were implanted over frontal cortex and cerebellum paying attention not to damage the pial membrane. EEG electrodes and skull were then wholly covered by dental cement. After the surgery, mice were returned to their home cage and left undisturbed for 24 h of recovery prior to the first rotarod session.

Complex wheel task. We modified the classical complex wheel task²⁶⁻³⁰ by attaching a complex wheel to an individual accelerating rotarod system (EZRod, Omnitech Electronics, Inc.) (Fig. 3A). To create a “complex” wheel, we used a running wheel that originally had 50 rungs, with rungs spaced 1.12 cm apart (wheel diameter 17.78 cm). These features are comparable to those of complex wheels previously used³⁰ whose diameter, number of rungs and space between rungs were 12.7 cm, 38 and 1.05 cm, respectively. We removed 20 rungs to make 2 identical complex sequences of rungs in one rotation (Fig. 3A). Prior to the first training all mice were weighed. At the beginning of the first session (20 trials), a mouse was placed onto the stationary complex wheel, and acceleration increased from 0 to 40 rpm over the course of 10 min (acceleration = 223.3 cm/min²). To encourage the mouse to keep running on the top of the wheel, a fluffy sponge was placed in the back above the wheel with a small space (1-2 cm, depending on the body size of the mouse) between the wheel and the sponge (Fig. 3A and Supplementary Movie). Mice did not receive any habituation or pretraining using the complex or the regular wheel, and thus usually spent some time exploring the device at the beginning of the first training session. If mice tried to escape from the chamber by grabbing the large disk connecting the rotarod to the motor system or by climbing up the sponge, they were gently placed back on top of the wheel. Mice sometimes also sniffed the sponge and squeezed their body below the sponge intentionally. In this case the trial was stopped and repeated. These events were rare and occurred

200 mostly at the lowest speed of the wheel (0~2 rpm). When the mouse could not keep up with the speed,
201 the body was squeezed in the tiny space between the sponge and the wheel, and the trial was manually
202 stopped by the experimenter by placing a hand in front of the infrared beam at the bottom of the
203 chamber. In most cases after each trial the mouse came back to the top of the wheel voluntarily,
204 suggesting that the task was not stressful (Supplementary Movie). After the first 10 trials mice were
205 returned to their home cage for a 5 min rest period, during which they mainly groomed but never slept.
206 Based on the median of the average performance in the first training session, mice were divided in fast
207 and slow learners and the effects of sleep and sleep deprivation were analyzed separately in each group,
208 consistent with studies in humans ³⁶. To test the importance of complex sequences in learning we also
209 used a regular 50 rungs wheel as a control. Four mice received the regular wheel task according to the
210 same protocol as the complex wheel task, with 2 sessions comprising 20 trials each, spaced 24h apart.
211 The acceleration profile was 0 to 40 rpm over the course of 10 min. A fluffy sponge was also placed in
212 the back above the wheel and each trial was manually stopped when the mouse was squeezed in the
213 space between the sponge and the wheel.

214

215 **Immunohistochemistry.** The immediate early gene c-fos is a marker of neuronal activation, although
216 the relationship between spontaneous neuronal activity and c-fos expression is not straightforward ³⁷.
217 Many regions of the brain contain a large number of Fos positive cells after animals have been awake
218 for as few as 1-2h, while after several hours of sleep Fos protein levels are undetectable in most,
219 although not all, neurons ³⁸. To focus on task-specific neuronal activity we aimed at reducing wake-
220 related Fos expression by allowing mice to sleep for several hours. Specifically, mice were confirmed
221 to have slept for more than 65% of the last 3h and 85% of the last hour before the perfusion (sleep
222 mice) or prior to the onset of training in the rotarod or complex wheel task (trained mice). Task
223 training occurred between 5:30PM and 7:15PM and each mouse was immediately killed after the task.
224 Mice were deeply anesthetized with isoflurane (3-5%) and transcardially perfused with a flush of
225 saline followed by 0.1 M phosphate buffer containing 4% paraformaldehyde. The brain was removed
226 and postfixed in the same fixative overnight at 4°C. The brain was then cut into 40 µm sections using a
227 vibratome and tissue sections were subjected to immunohistochemistry or kept in 0.05 M phosphate-
228 buffered saline (PBS) containing 0.05% sodium azide at 4°C until use. The sections were rinsed with
229 PBS and then incubated in PBS containing 0.1% hydrogen peroxide for 30 min to inactivate
230 endogenous peroxidases. After rinsing with PBS, the sections were incubated in blocking solution

(PBS containing 3% normal goat serum and 0.1% triton X-100) for 1 hr and then overnight in blocking solution containing the primary antibody against c-fos (sc-52; Santa Cruz Biotechnology, Santa Cruz, CA). The sections were subsequently reacted with a biotinylated secondary antibody (BA-1000; Vector Laboratories, Burlingame, CA) for 2 hr and visualized using the avidin-biotin system (PK-4000; Vector Laboratories) and diaminobenzidine (SK-4100; Vector Laboratories). Sections were rinsed 3 times between each reaction and all steps were done at room temperature. The sections were then dehydrated, coverslipped and examined under a light microscope. To analyze Fos expression, each brain region of interest was first identified based on the Allen Mouse Reference Brain Atlas. Specifically, for each coronal section and area of interest (e.g., anterior cingulate, primary motor, primary somatosensory) we measured on the Atlas medio-lateral and dorso-ventral extent, the latter subdividing the cortex in layers (layer 1, layers 2/3, layer 4 if applicable, layers 5/6). We then created a region-of-interest mask based on these measures and applied it to each of our images to identify the borders of each cortical area. Cortical depth (from layer 1 to the white matter below layer 6) as measured using the Atlas matched well that of our sections, so that we could designate each area consistently as shown in Figure 4b. Within each designated cortical area we then manually counted all Fos positive cells. The caudate-putamen was subdivided in 2 parts (medial and lateral) and cell counting was done separately for each of them. In the hippocampus, Fos positive cells were counted in CA1, CA3 and dentate gyrus and their number was expressed per length (in millimeters) of each hippocampal region.

250

Statistics. Data are expressed as mean values \pm SEM. All datasets were subjected to Shapiro-Wilk test to examine normality of distribution prior to each statistical analysis. Statistics were calculated by using paired or unpaired two-tailed Student's *t* test, one-way ANOVA with a post-hoc Tukey test, two-way repeated measures ANOVA with a post-hoc Bonferroni test, linear regression test, analysis of covariance, Pearson test or Spearman rank test, with IBM SPSS statistics 22. Student's *t* test and Pearson test were used for datasets with normal distribution and Spearman rank test was used for datasets with non-normal distribution. ANOVA was used in most statistical analyses based on its robustness against violation of normal distribution ³⁹.

259

260

261 **Results**

262 **Assessment of rotarod task and definition of measures of performance.** First, we used a training
263 routine employed in previous studies²². Specifically, 1 month-old YFP-H mice (n=7) were trained in
264 forward rotarod running (Fig. 1A, left) in 2 morning sessions, S1 and S2, spaced 24h apart. Between
265 sessions mice could sleep ad libitum. Each session included 40 trials, with the rod accelerating from 0
266 to 100 rpm over the course of 3 min²². Figure 1B shows the changes in performance in one
267 representative mouse across the first (S1) and the second (S2) session. Within each session there was
268 some variability from one trial to the next, and performance in the last trials tended to decrease and to
269 be more variable, perhaps due to fatigue. Since mean performance measured by averaging all trials in a
270 session does not fully capture variability and fatigue, we also measured performance across the first 3
271 trials (First), the best 3 trials (Max) and the last 3 trials (Last). Moreover, we used the ratio between
272 average performance in S2 and S1 (S2 Mean / S1 Mean) to calculate the performance improvement
273 across sessions, and the ratio Max / First in each session to assess intrasession improvement. Finally, to
274 test for offline, across sessions consolidation, we used 2 measures, S2 First / S1 Last and S2 First / S1
275 Mean. The first measure represents the most direct comparison of performance before and after sleep,
276 while the second measure controls for inter-trial variability and the potential issue of fatigue at the end
277 of the session. Both measures were used to assess offline consolidation within and across groups.

278
279 **No effects of sleep in the consolidation of the rotarod task using various experimental conditions.**
280 In the first experiment we compared the performance of mice that could sleep between the 2 sessions
281 with that of mice that were sleep deprived by gentle handling for 7h following S1 (7 mice/group; Fig.
282 1C). Similarly to a previous study²², mice of both groups improved in S2 relative to S1. However,
283 contrary to the previous report, we found no difference between the 2 groups in any of the parameters
284 that were assessed, including the overall profile of the learning curve (Fig. 1D,E), Mean, First, Max
285 and Last performance in each session (Fig. 1F-K). Most crucially, neither group showed evidence of
286 offline consolidation (Fig. 1J).

287 In a second experiment (Fig. S1A) one sleep group (n=7 mice) was compared to 2 SD groups,
288 one kept awake by gentle handling (SDgh, n=5), and the other by exposure to novel objects (SDob,
289 n=5), which in our experience is a more physiological and effective method of SD^{32, 35}. We reasoned
290 that in the first experiment with 40 trials, mice may have learned the task well enough to mask a clear
291 effect of sleep loss. Thus, in this experiment each session was limited to 20 trials. Time of training and
292 duration of sleep deprivation instead were not changed (Fig. S1A). Again, all 3 groups improved their

293 performance over the course of training, with no differences across groups in any of the examined
294 parameters (Fig. S1B-F), although in the SDob group mean offline consolidation reached significance
295 (Fig. S1F).

296 So far, all experiments used a fast acceleration profile, from 0 to 100 rpm in 3 min, which is the
297 same used in a recent study²² but faster than the one employed in other reports^{20, 40}. Thus, we also
298 trained mice using a slower acceleration profile (from 0 to 80 rpm in 5 min). Moreover, mice were first
299 trained at 8AM, as usual, but S2 occurred immediately after 7h of either SD (SDgh, n=3 or SDob, n=4)
300 or undisturbed sleep (n=4), to evaluate more immediate effects of sleep loss on learning (Fig. S1G).
301 Again, all mice improved their performance (Fig. S1H-L), and in fact, mean improvement across
302 sessions was significantly greater after SDob than after sleep (Fig. S1J) and offline consolidation was
303 larger in either SD group than in the sleep group (Fig. S1L), possibly because mice tested immediately
304 after SD were more alert and vigilant due to the stimuli used to keep them awake. Notably, despite the
305 slower acceleration profile, performance measures in all 3 groups were comparable to those in mice
306 that received training with the higher acceleration profile.

307 In the previous study, mice underwent surgery for EEG recording and two-photon imaging and
308 the first rotarod training was given 24h later²², when recovery from anesthesia and surgery may have
309 been incomplete. Since this condition of “stress” may have helped to unmask the negative effects of
310 SD, 2 other groups of mice underwent surgery for implant of EEG electrodes and 24h later received
311 the first session of rotarod practice. Afterwards, they were again divided into a sleep group (n=3) and
312 an SD group (n=3, Fig. S1M). Despite the surgery, we found no differences in performance between
313 the 2 groups, or their measures of learning and consolidation were in the range of those of intact mice
314 (Fig. S1N-R).

315 Mice are nocturnal, and tend to be asleep mostly during the day and be awake spontaneously
316 mostly during the night. Thus, in another experiment we assessed the effects of spontaneous wake by
317 scheduling the first training session at the end of the light phase, followed by S2 24h later (Fig. S2A).
318 As expected, in the dark period immediately following S1 mice spent the majority of the time awake
319 (wake as % of total time, 64.0 ± 1.9 in the first 4 h, 60.2 ± 2.4 in the first 7h after the end of training).
320 Overall levels of performance in S1 and improvement in S2 did not differ from those seen in the
321 sleeping mice used in the previous experiments (Fig. S2B-F). Thus, in our experimental setup
322 improvement in performance in the rotarod task occurred with a similar time course, and to the same
323 extent independent of whether after the first training mice were asleep, forced to stay awake, or

324 spontaneously awake. Moreover, this improvement in performance was present in all groups when
325 comparing mean speed across sections. By contrast offline consolidation (S2 First / S1 Mean) was
326 rarely seen: in fact, it was not observed in any of the sleep groups and was present only in one SD
327 experiment, when mice were tested immediately after sleep deprivation (Fig. S1L).

328

329 **No effects of sleep in learning the rotarod task or in the consolidation of the task in the presence**
330 **of interference.** To determine whether sleep loss may affect the ability to learn the rotarod task, rather
331 than impair the consolidation process following learning, we performed 7 h of SD prior to S1 (pSD,
332 Fig. S2G). Overall performance in S1 was slightly better in the pSD mice (n=4) relative to the sleeping
333 controls (n=7, Fig. S2H), although the difference did not reach statistical significance (Fig. S2I; Sleep*
334 S1 = 33.09 ± 2.45 rpm, pSD S1 = 38.63 ± 3.01 rpm). By contrast, performance improvement across
335 sessions was significantly lower in the pSD group, likely due to the high performance in S1 (Fig. S2J).
336 Overall, all performance measures in S2 did not differ between the 2 groups (Fig. S2I,K,L).

337 Next, we tested whether the consolidation of forward training would be impaired when
338 backward training occurred just a few hours after the first session of forward running, presumably
339 interfering with its consolidation. Since human studies suggest that sleep may help consolidation
340 especially in conditions of interference³, we reasoned that this protocol may help unmasking the
341 negative effect of sleep loss that we were unable to detect so far. Thus, 2 groups of mice were used: the
342 sleep group (n=5) slept for ~ 4 h after forward learning, then received backward training and was
343 allowed to sleep again ad libitum, while the sleep deprived group (n=6) was kept awake between
344 forward and backward training and for 2 h after backward training (Fig. S2M). As in a previous study
345²², backward training was implemented by using an anti-flipping tool that forced mice to run in the
346 “wrong” direction (Fig. 1A, right). We found no evidence that backward training interfered with the
347 consolidation of forward running, even when it was associated with sleep loss. Again, all mice learned,
348 and motor learning and performances in all measures did not differ between the 2 groups (Fig. S2N-R)
349 and were comparable to those seen in our previous experiments with forward training only. Therefore,
350 we didn’t find any deteriorating effects of SD in the rotarod task even when SD preceded S1 or was
351 coupled with interference.

352 To increase statistical power we also plotted all the data from experiments that shared the same
353 number of trials, 40 (Fig. 2A) or 20 (Fig. 2B), but still found no evidence for any change between the 2
354 groups in the time course of performance improvement, either within or across sections. We then

355 tested the relationship between mean and late performance in S1 and mean and early performance in
356 S2 using data from all the mice (Fig. 2C,E,G). Large interindividual variability was present, but there
357 was also a highly significant correlation, in all the groups, between performance in S1 and S2. Thus
358 independent of sleep, high performance during the first training was more likely associated with high
359 performance in the following session (Fig. 2D). Note also that offline gains, measured by comparing
360 the performance at the beginning of S2 (S2 First) with either the average or last performance of S1 (S1
361 Mean or S1 Last), were not present in the sleep group but occurred in SD mice (Fig. 2F,H). This gain,
362 however, was driven by the SD mice of one single experiment (Fig. S1G-L).

363 To understand why we could not replicate the results of the previous study that found beneficial
364 effects of sleep in rotarod performance, we estimated performance means during the first training
365 session in the mice of that study (based on their Figures 3C and S5)²²) and compared them with those
366 of our mice. Mean performance in S1 was 32.2 rpm for their sleep mice (n=5), which is very similar to
367 that in our sleep mice (see Figure S3A), while their SD mice (n=7) had a mean performance in S1 of
368 22.4 rpm, a value that is lower than ours (Fig. S3A). Thus, SD mice in the previous study may have
369 been on average poor performers, and performance in the 2 groups may not have been well balanced.
370 Yet, in our own data we found a strong correlation between mean performance in S1 and S2 (Fig. 2C),
371 but not between mean performance in S1 and overall improvement across sessions (Fig. S3B). Thus,
372 mice with low performance in S1 do not necessarily show low performance improvement across
373 sessions. In summary, we do not have any obvious explanation for the discrepancy, but laboratory
374 environment affects mouse behavior, and there may be subtle differences in the way the same task is
375 implemented across laboratories^{41,42}. Finally, rotarod performance in mice was previously shown to
376 be negatively correlated with body weight^{43,44}, while we found no correlation between body weight
377 and motor performance (Fig. S3C). However, our mice were smaller (13~21 g) and our training
378 protocol (40 trials) was more demanding than in previous studies, which used one single⁴³ or three
379 trials per day⁴⁴. Thus, intense learning may have masked any effect of weight. There is also conflicting
380 evidence about sex differences in rotarod performance^{45,46}, but in our experiments males and females
381 performed at similar levels (Fig. S3C).

382

383 **Sleep consolidated motor learning in the complex wheel task.** Next we tested whether sleep
384 facilitates the consolidation of complex motor skills that include sequence learning. To this aim we
385 developed a modified version of the complex wheel task by attaching a complex wheel to the device

386 used to run the rotarod task (Fig. 3A and Supplementary Movie). As described in the Methods section,
387 our version differs from the classical complex wheel task²⁶⁻³⁰ in that mice are forced to run on top of
388 the wheel rather than inside. To increase the chance to see sleep-dependent effects mice were not
389 pretrained, and intense training occurred within a limited time frame. Specifically, each training
390 session contained 20 trials and the acceleration was 0 to 40 rpm over the course of 10 min. The
391 measures of performance were the same used in the rotarod experiments, to compare the results
392 obtained with the 2 tasks (Fig. 3B).

393 In the first experiment mice received the first training at 8AM and were then divided into a
394 sleep group and an SD group that was kept awake by gentle handling for 7 h starting immediately after
395 S1. All mice received S2 at 8AM the next day (Fig. 3C, Morning- to-morning paradigm). Studies in
396 humans found large inter-individual variability in learning motor tasks and differential effects of sleep
397 in fast and slow learners³⁶. From the very beginning of the study we noticed that our mice also varied
398 widely in their ability to perform the task. Thus, consistent with studies in humans, we used the median
399 of the average performance in S1 to divide the mice in fast and slow learners, and studied the effects of
400 sleep separately in the 2 groups (Fig. 3D). We first describe all the results for the fast learners and later
401 (Fig. 5) discuss the slow learners.

402 Among the fast learners in the morning-to-morning paradigm, sleep mice showed higher
403 performance in S2 than SD mice, especially in the first half of the session (Fig. 3D,E). Specifically,
404 sleep mice had higher mean performance (Fig. 3F), higher performance improvement across sessions
405 (Fig. 3G,H) and higher first and max performance (Fig. 3I) than SD mice. Crucially, sleep mice, but
406 not SD mice, were also significantly better at the beginning of the second session relative to their own
407 mean performance in the first session (ratio S2 First / S1 Mean), resulting in a significant difference
408 between the 2 groups (S2 First / S1 Mean, Fig. 3J). Results using the second measure of offline
409 consolidation showed a similar trend, which however did not reach significance (S2 First / S1 Last; $p =$
410 0.116, Student's t test; Fig. 3J). Intrasession improvement instead was not significantly different
411 between the 2 groups (Fig. 3K). Of note, performance improvements were not found when another
412 group of mice ($n=4$) run on a regular wheel without any pretraining: in this case, mice showed high
413 performance (~10 rpm) from the very beginning of the first training session without any improvement
414 across trials (Fig. S4A-C), or across sessions (Fig. S4D). Maximal performance in S1 (S1 Max) was
415 not significantly different from initial performance (S1 First) (Fig. S4E), indicating lack of intrasession
416 improvement.

417

418 **Sleep-dependent consolidation in the complex wheel task confirmed in same day paradigms.** To
419 test whether sleep-dependent consolidation in the complex wheel task occurs within a few hours after
420 the first training session other groups of mice received S1 at 8AM and S2 immediately after 7 h of
421 either sleep or sleep deprivation by gentle handling (Fig. S5A, Morning-to-afternoon paradigm). In this
422 case, fast learners of both groups showed very similar performance in both sessions, in all measures
423 (Fig. S5B-I). We noticed, however, that some sleep mice appeared drowsy at the beginning of S2, most
424 likely because their sleep was abruptly terminated to start S2, suggesting that as in humans, sleep
425 inertia may have masked the beneficial effects of sleep⁴⁷⁻⁵⁰. Consistent with this hypothesis, in the
426 sleep group we found a positive correlation between time spent awake during the last hour before S2
427 and either performance improvement across sessions or S2 Mean performance (Fig. S5J,K). This
428 positive correlation was not found using the previous morning-to-morning paradigm (Fig. S6A-C).

429 To avoid sleep inertia in the next experiment sleep mice were allowed to sleep 9 h, instead of 7
430 h, and had 30 min of exposure to novel objects prior to S2 (Fig. 4A, Morning-to-late afternoon
431 paradigm). SD mice were kept awake by exposure to novel objects for the same amount of time (9.5 h).
432 Using this study design, sleep mice did not appear drowsy at the onset of S2, and we found no
433 correlation between time spent awake prior to S2 and performance in S2 (Fig. S6D-F). Consistent with
434 the morning-to-morning experiment, among the fast learners sleep mice showed higher performance
435 than SD mice in all S2 measures (Fig. 4B-G). Moreover, sleep mice showed significant offline
436 consolidation, both relative to their own performance in S1 and as compared to SD mice, and did so
437 using both measures of offline consolidation (Fig. 4G).

438 Next, to exclude the possibility that SD mice showed lower performance because of fatigue we
439 left all mice undisturbed for ~5 h after 7 h of sleep or SD by gentle handling, and performed S2 1 h
440 after lights off (Fig. 4H, Morning-to-night paradigm). Fast learners of both groups showed similar
441 amount of spontaneous wakefulness just prior to S2 (Fig. S6G-I), ruling out the possibility that SD
442 mice were sleepy even in the dark phase due to the sleep loss in the previous light phase. Also with this
443 paradigm we found that sleep mice showed in S2 higher performance than SD mice in all measures
444 (Fig. 4I-N). Moreover, sleep mice again showed significant offline consolidation as compared to SD
445 mice using both measures (Fig. 4N).

446

447 **Sleep consolidates motor skill of the complex wheel task differently in fast and slow learners.**

448 Next, we studied the effects of sleep on slow learners and compared them to those already described
449 for the fast learners. To obtain a large and balanced number of animals in each group (fast vs. slow,
450 sleep vs. SD) we pooled the data from all the experiments except the morning-to-afternoon paradigm,
451 whose results were confounded by sleep inertia. First, we tested whether at least some of the inter-
452 individual variability was due to differences in body weight and/or gender, and found that it was not
453 (Fig. S7).

454 Among the fast learners, there were 40 mice in the sleep group and 36 mice in the SD group
455 (Fig. 5A). In both groups performance in S1 predicted performance in S2 (linear regression analysis,
456 sleep mice, $R^2 = 0.28$, $F(1,38) = 14.773$, $p < 0.001$; SD mice, $R^2 = 0.27$, $F(1,34) = 12.30$,
457 $p < 0.01$). Moreover, both groups improved in S2 relative to S1, but sleep mice did so more than SD
458 mice (Fig. 5B). Crucially, sleep mice showed offline consolidation when compared to SD mice.
459 Specifically, at the onset of S2, sleep mice as a group maintained, but did not exceed, the peak
460 performance reached at the end of S1, perhaps because they had already reached the highest scores
461 afforded by a single training session (Fig. 5C,D). Performance in SD mice, on the other hand, was
462 significantly worse at the onset of S2 than at the end of S1 (Fig. 5C,D), suggesting that sleep is
463 required to prevent performance decay. Mean performance in S2 was positively correlated with time
464 spent asleep during the 7h after S1, while mean performance in S1 did not predict subsequent sleep
465 quantity (Fig. 5E). Moreover, time spent asleep after initial training was positively correlated with one
466 measure of offline consolidation (S2 First / S1 Mean), although not with the other (S2 First / S1 Last)
467 (Fig. 5F), again perhaps due to a ceiling effect.

468 The slow learners included 42 sleep mice and 33 SD mice (Fig. 5G). Performance in S1
469 predicted performance in S2 only in sleep mice but not in SD mice (linear regression analysis, sleep
470 mice, $R^2 = 0.25$, $F(1,40) = 7.062$, $p < 0.05$; SD mice, $R^2 = 0.05$, $F(1,31) = 1.583$, $p > 0.05$). Still,
471 both groups improved in S2 relative to S1 (Fig. 5H). Slow learners also showed evidence of offline
472 consolidation after sleep when compared to after sleep deprivation, but for reasons different from those
473 seen in the fast learners. Specifically, at the onset of S2 sleep mice as a group showed an offline gain,
474 that is they exceeded the peak performance reached at the end of S1 (Fig. 5I,J). Unlike in the fast
475 learners, however, sleep deprivation did not lead to performance decay at the onset of S2 (Fig. 5I,J). In
476 contrast to fast learners, time spent asleep after initial training did not correlate with measures of
477 offline consolidation or mean performance in S2 (Fig. 5K,L).

478

479 **Complex wheel training activates more neurons in motor cortex and hippocampus than rotarod**
480 **training.** Both the complex wheel task and the rotarod task require the mice to run on an accelerating
481 device, but in the former the mouse needs to learn complex movement sequences and relies more on
482 the use of fine movements and visuo-spatial coordination. Thus, the 2 tasks are expected to rely on
483 partially different patterns of neuronal activation. To identify them, we used Fos as marker of neuronal
484 activity. To perform Fos immunohistochemistry mice were perfused immediately following the first
485 training session (Fig. 6A). Since wake is associated with widespread increased expression of Fos
486 relative to sleep, all mice were allowed to sleep for several hours before the task, to eliminate previous
487 wake-related Fos expression^{37,38}. Moreover, since mice take roughly half of the time to perform the
488 same number of trials in the rotarod task relative to the complex wheel task, we compared animals that
489 received 20 or 40 trials of rotarod training to those that received 20 trials of complex wheel training.
490 Fos positive cells were manually counted in the medial prefrontal cortex (prelimbic and anterior
491 cingulate areas), primary and secondary motor cortices, primary somatosensory cortex, striatum and
492 hippocampus (Fig. 6B).

493 As expected, sleep controls showed negligible Fos expression in most of the brain regions (Fig.
494 6B-F). In all tested regions, mice that received 20 trials of rotarod training exhibited less Fos positive
495 cells than the other trained mice (Fig. 6B-F), probably because of the shorter awake time (Fig. 6G).
496 Thus, we focused on the comparison between mice that underwent 40 trials of rotarod training and
497 mice that received 20 trials of complex wheel training (all fast learners), as total awake time was
498 similar in these 2 groups (Fig. 6G). Compared to rotarod training, complex wheel learning led to a
499 significantly higher number of Fos positive cells in supragranular and infragranular layers of primary
500 motor area (Fig. 6E) and of secondary motor area (Fig. 6C,D), as well as in the CA1 region of the
501 hippocampus (Fig. 6B,F). By contrast, no significant differences between the 2 groups were found in
502 prelimbic and anterior cingulate cortex, dorsomedial and dorsolateral striatum, primary somatosensory
503 cortex, CA3, and dentate gyrus of the hippocampus (Fig. 6C-F).

504

505 **Discussion**

506 Sleep-dependent consolidation of motor skills is well documented in humans, but much less so in
507 animals. One of the few studies in mice recently suggested that sleep loss affects the consolidation of
508 rotarod learning²². One of our goals was to build on these results and refine the evidence for offline

consolidation. To follow the previous study as closely as possible, we used mice of the same transgenic line and age, as well as the same rotarod system and experimental design as reported previously²². However, to our surprise, mice improved equally well after sleep and after SD, independent of the method of SD (gentle handling vs. novel objects), time of testing (second training immediately after SD vs. the next day), length of training (20 vs. 40 trials), and whether or not they had undergone surgery 24h before training. We also found that mice that were trained at the end of the light phase and then remained spontaneously awake for several hours improved as much as mice trained during the day and allowed to sleep after practice. For the first time, we also tested the effects of SD performed before the first training session, as well as the effects of SD in mice trained in a more complex paradigm that involved forward running followed by backward running. In both experiments sleep deprived mice and sleeping controls performed equally well. Overall, there was no difference in mean performance between SD mice and sleeping controls in any of the 7 experimental designs we employed. For the first time we also directly tested whether there was an offline gain in performance – sleep-dependent consolidation – by comparing performance at the beginning of the second session (S2 First) with either the last or the mean performance of the first session (S1 Last or S1 Mean). We found no evidence for better consolidation in mice allowed to sleep ad libitum either for 7h or until the next day. If anything, we found some evidence for offline consolidation in a subset of SD mice, but this effect was limited to a single experiment. Finally, we found large interindividual variability in the way sleep and sleep loss affected this task. Thus, we conclude that sleep does not benefit motor learning in the rotarod task (Table S2), contrary to a previous report that was based on a small number of animals.

The complex wheel task demands close attention to the sequence of uneven rungs which would serve as complex cues for learning and requires complex movements of limbs and paws with high spatial accuracy. Therefore, it is perhaps not surprising that we found higher Fos expression, and thus presumably stronger neuronal activation, in a few select areas after complex wheel training compared to rotarod training. These areas included the supragranular and infragranular layers of primary motor cortex, the same layers that undergo plastic changes in response to training in the reaching task, including LTP-like strengthening of cortical connections and spine formation^{51,52}. Higher Fos expression was also present in all layers of secondary motor cortex. This area in rodents is akin to the supplementary motor area of primates^{53,54}, which has an established role in planning, initiation and control of complex movements and motor routines^{55,56}. Consistent with our data, another study in humans showed that regional cerebral blood flow in the supplementary motor area increased more

during complex motor tasks than simple ones ⁵⁶, suggesting that the activity in this region reflects the complexity of the task. In our study, Fos expression was more pronounced in the rostral, compared to the caudal, part of secondary motor cortex (Fig. 6C-E), pointing to the former as the most critical area for learning or executing the complex wheel task. Moreover, a recent study in humans found that training in a finger tapping task led to an increase in sleep slow waves and fast spindles in the contralateral supplementary motor area, and these local sleep changes correlated with performance improvement ⁵⁷. Finally, Fos expression was also higher in the CA1 region of the hippocampus after complex wheel training relative to rotarod training (Fig. 6C-F). The hippocampus likely plays an important role in the initial phase of motor sequence learning, possibly because of its role in the promotion of higher order associations and processing of spatial information ⁸. In addition to motor complexity, the complex sequence of rungs might also serve in increasing cue complexity, which is another important entity given that a replay of sequential activity of place cells encoding environmental cues occurs during sleep and plays a critical role in sleep-dependent consolidation ^{58,59}. Moreover, some studies in humans have specifically linked the hippocampus to motor sequence learning ⁶⁰ and to the sleep-dependent consolidation of these tasks ^{8,9}. Thus, the strong involvement of both motor cortex and hippocampus in mice seem to support these conclusions.

A previous study in humans found that the overnight gain in performance after training in a motor sequence task was limited to fast learners and not found in slow learners ³⁶. The same study found that fast and slow learners recruited different neural systems during training - hippocampus and cerebellum, respectively - suggesting that sleep effects may also depend on the specific neural networks engaged during training. We found differential effects of sleep based on performance, although both fast and slow learners improved after sleep. In fast learners, sleep consolidated motor memory by stabilization, that is by preserving the skills learned during the first session. This result is in line with the evidence for sleep-dependent consolidation in rodents in various hippocampus-dependent tasks, including contextual fear conditioning ⁶¹⁻⁶³, radial arm water maze ^{64,65}, Morris water maze ⁶⁶, reversal learning of Y maze ⁶⁷ and novel object-place recognition ⁶⁸. Using these tasks sleep-dependent stabilization was documented both in mice ^{61,67} and rats ^{63-66,69,70}, since at the beginning of the retest session memory was impaired after sleep deprivation but preserved after sleep. We also found, however, that longer sleep correlated with one measure of offline gain, as well as with the mean performance in the second session. Thus, at retest, performance in our sleep and SD mice may have differed not only because of the deteriorating effects of SD, but also due to a direct positive effect of

571 sleep. Among the slow learners performance did not get worse after sleep loss, perhaps because it was
572 already low at the end of the first session. Sleep, on the other hand, led to an offline gain, although we
573 could not find any correlation between this effect and time spent asleep after initial training. One study
574 in humans found a correlation between offline gain in performance of motor sequence learning and the
575 amount of stage 2 NREM sleep specifically during the last quarter of the sleep period ². Thus, we may
576 have missed the correlation because we could only assess total sleep duration.

577 Our mice showed prominent inter-individual variability in absolute levels of performance and
578 performance improvement across sessions. The correlation between sleep and subsequent performance
579 in fast learners may account for some of the inter-individual variability among the S group. Still,
580 several sleeping mice showed little or no improvement, or even worse performance after sleep,
581 suggesting that sleep is only one of the factors affecting memory consolidation in this task. Different
582 from the previous studies giving mice free access to a complex wheel ²⁶⁻³⁰, our task requires manual
583 intervention to give mice an intense training. Therefore, different levels of psychological stress derived
584 from the inherent feature of the task might also contribute to the inter-individual variability because
585 stress may affect the whole process of motor learning, sleep, and consolidation. Also unclear are the
586 reasons for the inter-individual variability after sleep deprivation: more SD mice than sleep mice
587 showed lack of memory consolidation across sessions, but many SD animals performed at retest as
588 well as sleep mice. In humans, there are stable, trait-like differences in the susceptibility to cognitive
589 impairment caused by acute SD or chronic sleep restriction ⁷¹⁻⁷³, which are at least partially attributable
590 to genetic background ⁷⁴. Our mice, however, shared the same genetic background and thus other
591 factors must be involved. In humans, neuroimaging studies found that differences in the activation of
592 fronto-parietal regions during a working memory task at rest are associated with differences in the
593 extent of the cognitive decline during SD ^{75,76}. Moreover, recent evidence suggests that differences in
594 the microstructure of the white and grey matter can underlie the inter-individual differences in the
595 resistance to sleep loss ⁷⁷⁻⁷⁹. To our knowledge, there are no studies in sleep-deprived rodents focusing
596 on inter-individual differences and their underlying mechanisms.

597 In summary, our results show for the first time in mice that sequence learning benefits from
598 sleep, while rotarod training, an easier task that is associated with less pronounced activation of motor
599 cortex and hippocampus, does not. We also show for the first time in mice, where genetic factors are
600 easier to control, that the effects of sleep and sleep loss greatly vary from mouse to mouse. This
601 interindividual variability, which is increasingly being recognized in humans, strongly suggests that

602 factors other than sleep must modulate memory consolidation in the first crucial hours that follow
603 learning.

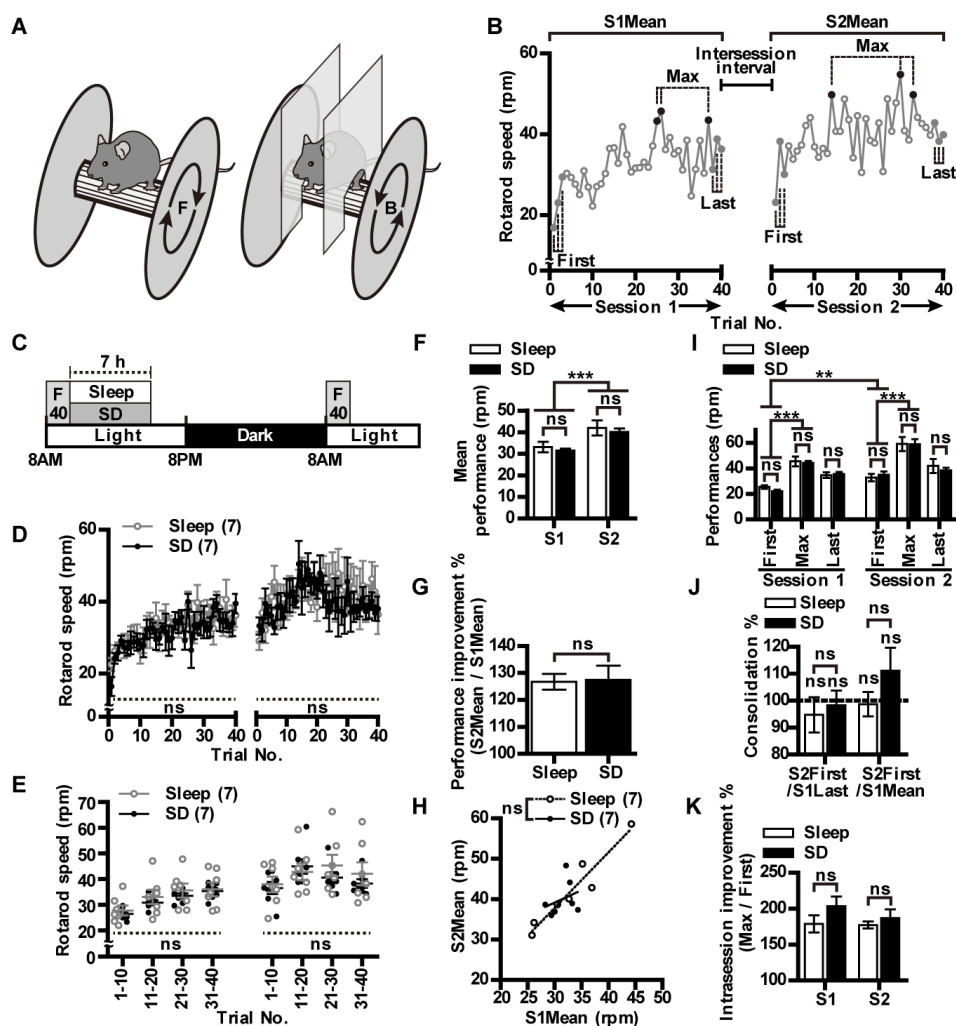
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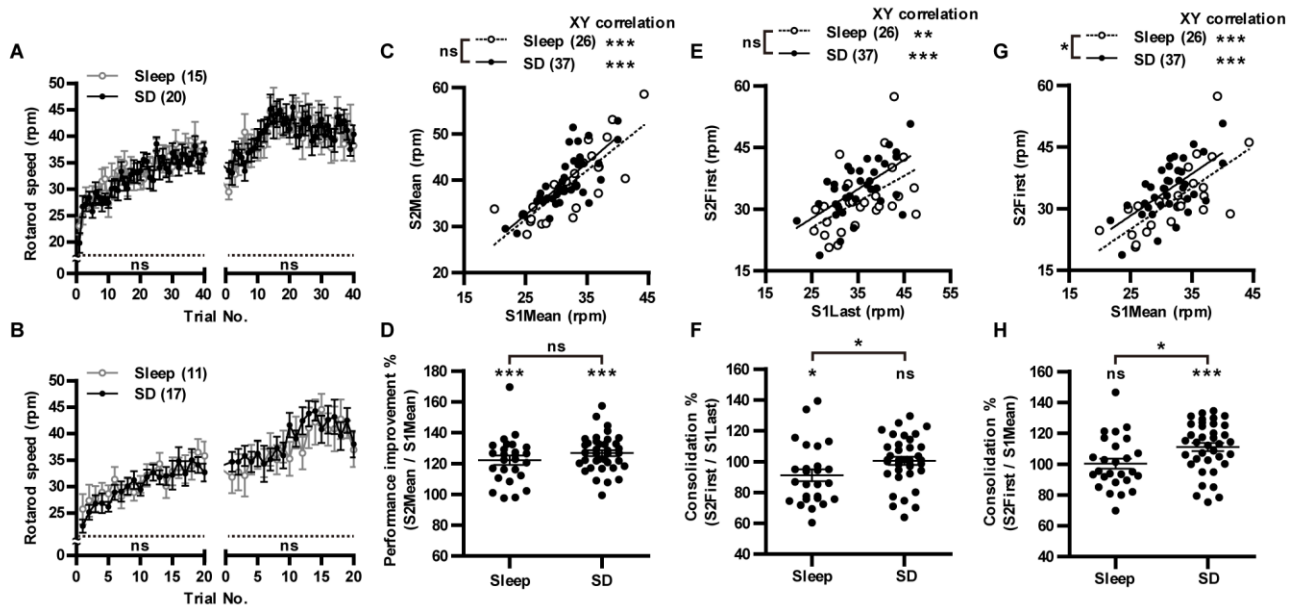
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610 **Figure 1. Rotarod task, measures of performance, and no evidence for sleep-dependent consolidation.** (A) Schematic
611 of the accelerating rotarod system with forward (F, left) and backward (B, right) running. In the backward running, the
612 mouse is prevented from switching body position by an anti-flipping tool. (B) Intra- and intersession changes in
613 performance in a single representative mouse, and the different parameters used to assess performance in each session: first
614 3, maximal 3, and last 3 trials, and mean of all trials. (C) Schematic of the experimental design. Mice were subjected to the
615 first session of rotarod training at 8AM (S1, 40 trials) and then divided in 2 groups (n=7 per group), depending on whether
616 in the following 7 h they could sleep or were sleep deprived (SD) by gentle handling. The next day starting at 8AM mice
617 were trained again (S2, 40 trials). (D) Performance values for each single trial after pooling all mice within each group. (E)
618 Performance values for each single mouse after pooling values in groups of 10 trials. (F) Mean performance for each
619 session. (G) Performance improvement across sessions. (H) Relationship between S1 Mean and S2 Mean for each mouse.
620 Statistical significance was calculated by comparing the linear regression lines of Sleep and SD. (I) Performance measures
621 for each session in the 2 groups. (J) Measure of offline consolidation. (K) Relative intrasession improvement. Values are
622 expressed as mean \pm SEM. **p<0.01, ***p<0.001; two-way repeated measures ANOVA followed by Bonferroni post hoc
623 test was used in (D-F,I,K), Student's *t* test in (G,J) and linear regression analysis followed by analysis of covariance in (H).
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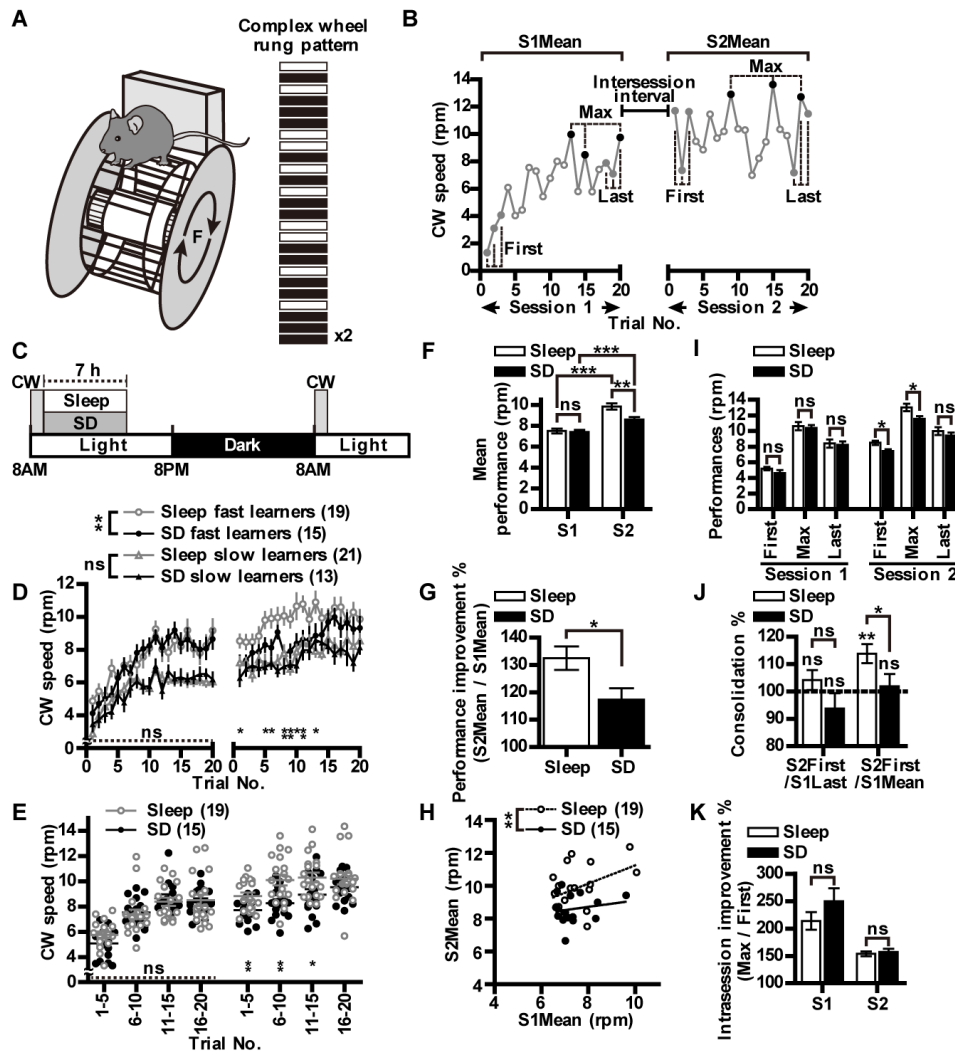


625

Figure 2. Overall analysis of rotarod learning. (A) Pooled data of all experiments with 40 trials (Fig. 1C, Fig. S1M, Fig. S2A,M). The experiment in which sleep deprivation was done prior to S1 is excluded. (B) Pooled data of all experiments with 20 trials (Fig. S1A,G). Statistical significance was calculated by comparing SD mice and sleeping controls in each session. (C,E,G) Relationship between S1 Mean and S2 Mean (C), S1 Last and S2 First (E) or S1 Mean and S2 First (G) for each mouse shown in A and B. Statistical significance was calculated by comparing the linear regression lines of sleep and SD. (D) Performance improvement across sessions for each mouse shown in A and B. Comparison between S2 Mean and S1 Mean within each group is indicated above each plot. (F,H) Consolidation of motor learning in each mouse assessed by using 2 measures, S2 First / S1 Last (F) and S2 First / S1 Mean (H). Comparison between S2 First and S1 Last or S1 Mean within each group is indicated above each plot. Values are expressed as mean \pm SEM. * $p < 0.05$, *** $p < 0.001$; two-way repeated measures ANOVA followed by Student's t test was used in (A,B), linear regression analysis, analysis of covariance and Spearman rank correlation test in (C,E,G), and Student's t test in (D,F,H).

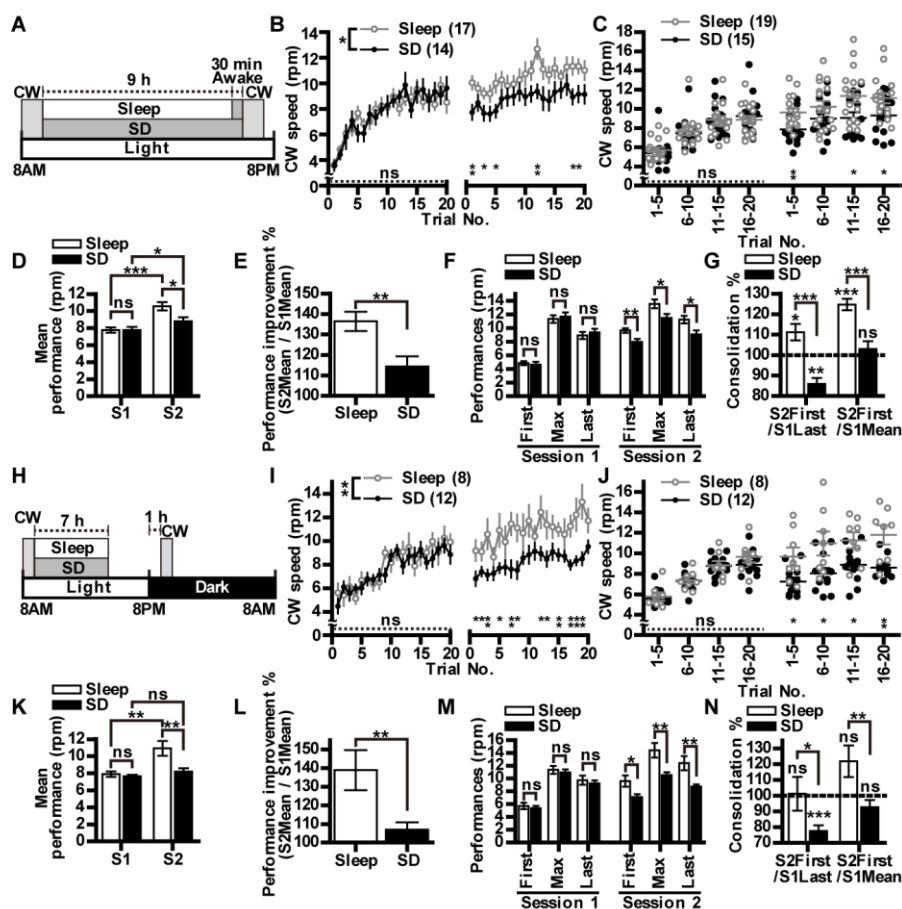


641 **Figure 3. Sleep-dependent consolidation of motor learning using the complex wheel task: next day experiments.** (A)
 642 Schematic and rung pattern of the complex wheel (CW). (B) Intra- and intersession changes in performance in a single
 643 representative mouse, and the different parameters used to assess performance in each session: first 3, maximal 3, and last 3
 644 trials, and mean of all trials. (C) Experimental design. After the first session (S1, 20 trials) at 8AM, mice were divided in 2
 645 groups depending on whether in the following 7 h they could sleep or were sleep deprived (SD) by gentle handling. The
 646 next day starting at 8AM mice were trained again (S2, 20 trials). (D) Performance of fast and slow learners in the sleep and
 647 SD groups shown for each single trial. (E) Performance in sleep and SD mice pooled across 5 trials; in this and the
 648 following panels, only data from fast learners are shown. (F) Mean performance for each session. (G) Mean performance
 649 improvement across sessions. (H) Relationship between S1 Mean and S2 Mean in each mouse. Statistical significance was
 650 calculated by comparing the linear regression lines of S and SD. (I) Performance measures for each session in the 2 groups.
 651 (J) Offline consolidation of motor skills using 2 measures. (K) Relative intrasession improvement. Values are mean \pm SEM.
 652 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-way repeated measures ANOVA followed by either Bonferroni post hoc test or
 653 Student's t test was used in (D-F,I,K), Student's t test in (G,J) and linear regression analysis followed by analysis of
 654 covariance in (H). ns, not significant.



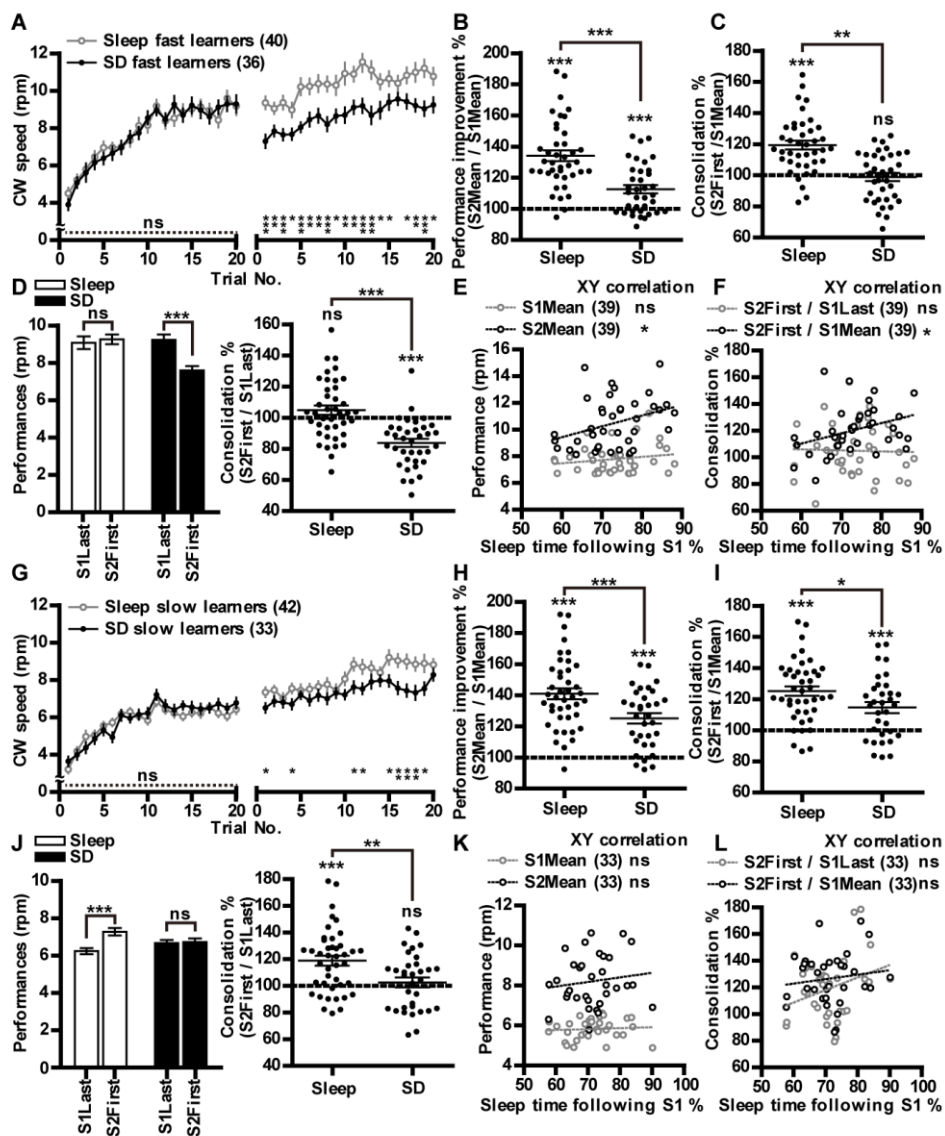
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656 **Figure 4. Sleep benefits motor learning in the complex wheel task: same day experiments.** (A) Experimental design for
657 the morning-to-late afternoon paradigm. After the first session (S1, 20 trials) at 8AM, mice were divided in 2 groups (Sleep
658 n=24; SD n=23) depending on whether they could sleep or were sleep deprived afterwards. Sleep mice were left
659 undisturbed for 9 h and received 30 min exposure to novel objects to dissipate sleep inertia, whereas SD group was
660 deprived of sleep for 9.5 h by novel objects. The same day starting at 6:30PM mice were trained again (S2, 20 trials). Only
661 fast learners are shown (slow learners, n = 7 Sleep mice; n= 9 SD mice are shown in Fig. 4). (B,C) Performance in the 2
662 groups shown for each single trial (B) and each 5 trials (C). (D) Mean performances for each session. (E) Performance
663 improvement across sessions. (F) Performance measures for each session in the 2 groups. (G) Consolidation of motor skills
664 using 2 measures. (H) Schematic of the experiment of the morning-to-night paradigm. Mice were subjected to the first
665 session (S1, 20 trials) of complex wheel task at 8AM and then divided in 2 groups (18 Sleep and 18 SD) depending on
666 whether in the following 7 h they could sleep or were sleep deprived by gentle handling. After 7 h, both groups were left
667 undisturbed until they were trained again the same day at 9PM (S2, 20 trials). Lights were always on in the training room.
668 Only fast learners are shown (slow learners, n = 10 Sleep mice; n= 6 SD mice are shown in Fig. 4). (I-N) Same measures as
669 in B-G. Values are mean \pm SEM. *p<0.05, **p<0.01, ***p<0.001; two-way repeated measures ANOVA followed by either
670 Bonferroni post hoc test or Student's *t* test was used in (B-D,F,I-K,M), and Student's *t* test in (E,G,L,N). CW, complex
671 wheel; SD, sleep deprivation; S, session; ns, not significant.

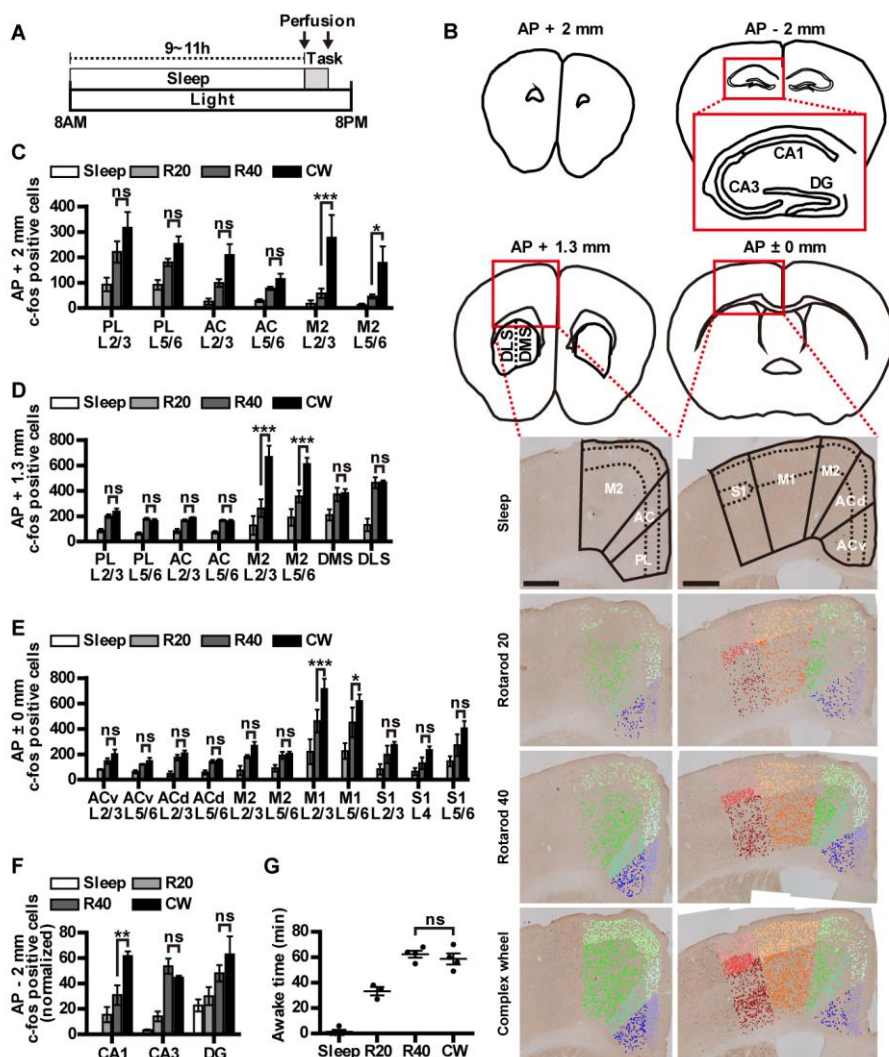


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674 **Figure 5. Comparison between fast and slow learners.** Data were pooled across 3 experimental paradigms (morning-to-
675 morning, to-late afternoon, to-night) of fast and slow learners. The threshold to define fast and slow learners is based on the
676 median of mean S1 performance across all pooled mice. (A-F) Fast learners. (A) Performance of each single trial. (B)
677 Performance improvement across sessions. (C) Offline consolidation using the S2 First / S1 Mean ratio. (D) Offline
678 consolidation using the S2 First / S1 Last ratio, with absolute performance values shown on the left panel. (E) Relationship
679 between sleep time during the 7h following S1 and mean performance of each session. Activity data of one mouse was
680 missing. (F) Relationship between sleep time following S1 and offline consolidation using 2 measures (S2 First/S1 Last and
681 S2 First/S1 Mean). (G-L) Same measures as in a-f for slow learners. Activity data of nine mice were missing in (K,L).
682 Values are mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Comparison within each group is indicated above each plot in
683 (B-D,H-J); two-way repeated measures ANOVA followed by Student's t test was used in (A,G), Student's t test in (B-D,H-
684 J), and correlation analysis was calculated in (E,F,K,L) either by Pearson or Spearman test based on normality of samples.
685 CW, complex wheel; SD, sleep deprivation; S, session; ns, not significant.

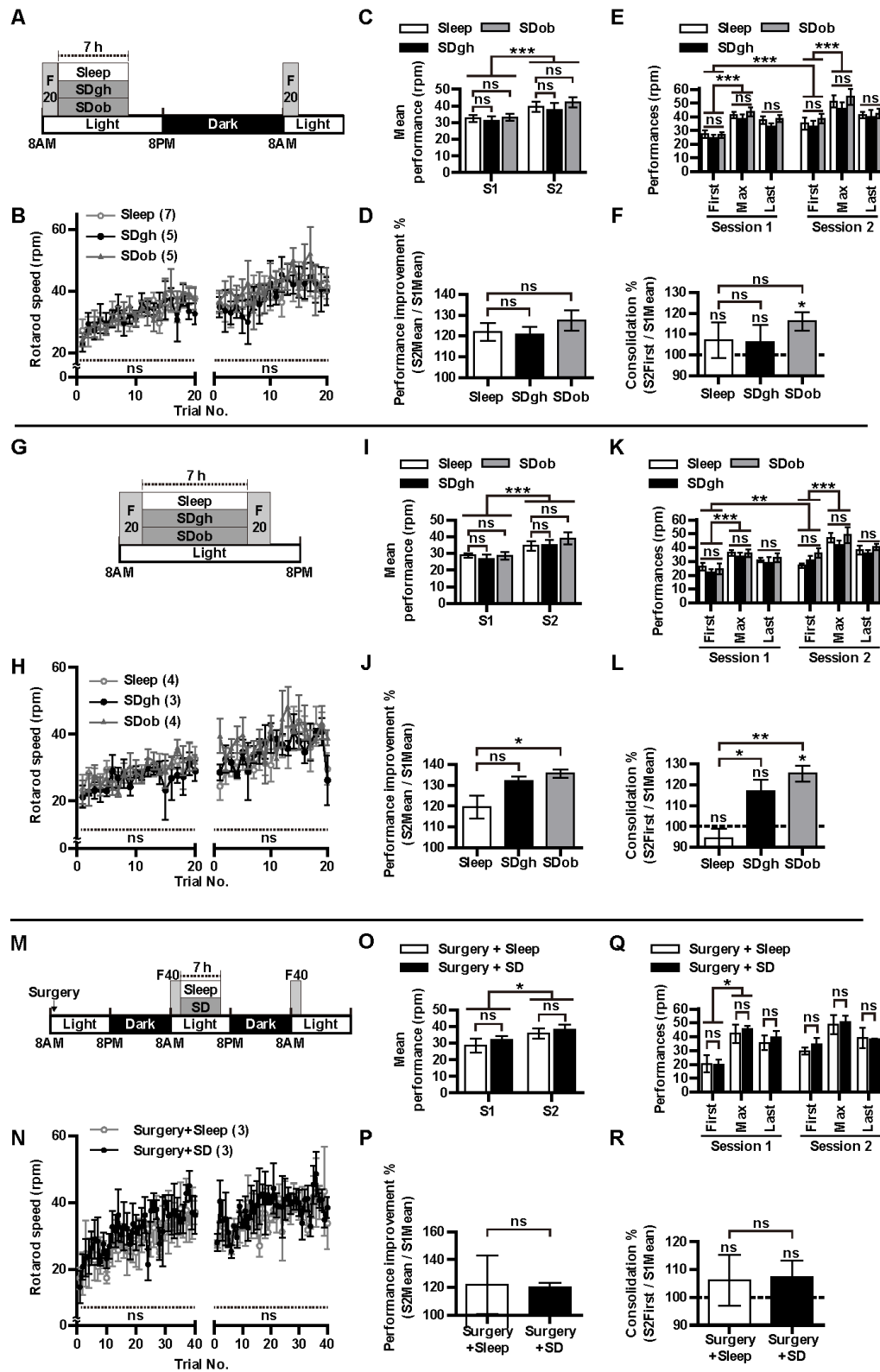


687 **Figure 6. Complex wheel training leads to differential Fos expression in select areas relative to rotarod training.** (A)
688 Experimental design. Mice were confirmed to have slept before they were subjected to either immediate perfusion (sleep
689 control, n=4) or motor task training (rotarod 20 trials, R20, n=3; rotarod 40 trials, R40, n=4; complex wheel 20 trials, CW,
690 n=4, all fast learners). (B) Schematics of each brain area analyzed and representative results of Fos immunohistochemistry.
691 The designated cortical area was determined based on the Allen mouse brain atlas. Each dot represents a Fos positive cell
692 identified by manual counting. Scale bars = 500 μ m. (C-F) Number of Fos positive cells in different brain areas
693 corresponding to bregma +2 mm (C), +1.3 mm (D), \pm 0 mm (E) and -2 mm (F) AP. (G) The duration between the time
694 when mice were taken out from their home cage and the time when perfusion occurred is shown as the awake time. In the 3
695 groups of trained mice, awake time is mostly the time spent on the task. Values are mean \pm SEM. * p <0.05, ** p <0.01,
696 *** p <0.001; two-way ANOVA followed by Bonferroni post hoc test was used in (C-F) and one-way ANOVA followed by
697 Tukey post hoc test was used in (G). PL, prelimbic area; ACv, anterior cingulate area ventral part; ACd, anterior cingulate
698 area dorsal part; M1, primary motor area, M2, secondary motor area; DMS, dorsomedial striatum; DLS, dorsolateral
699 striatum; S1, primary somatosensory area; DG, dentate gyrus; CW, complex wheel; ns, not significant.



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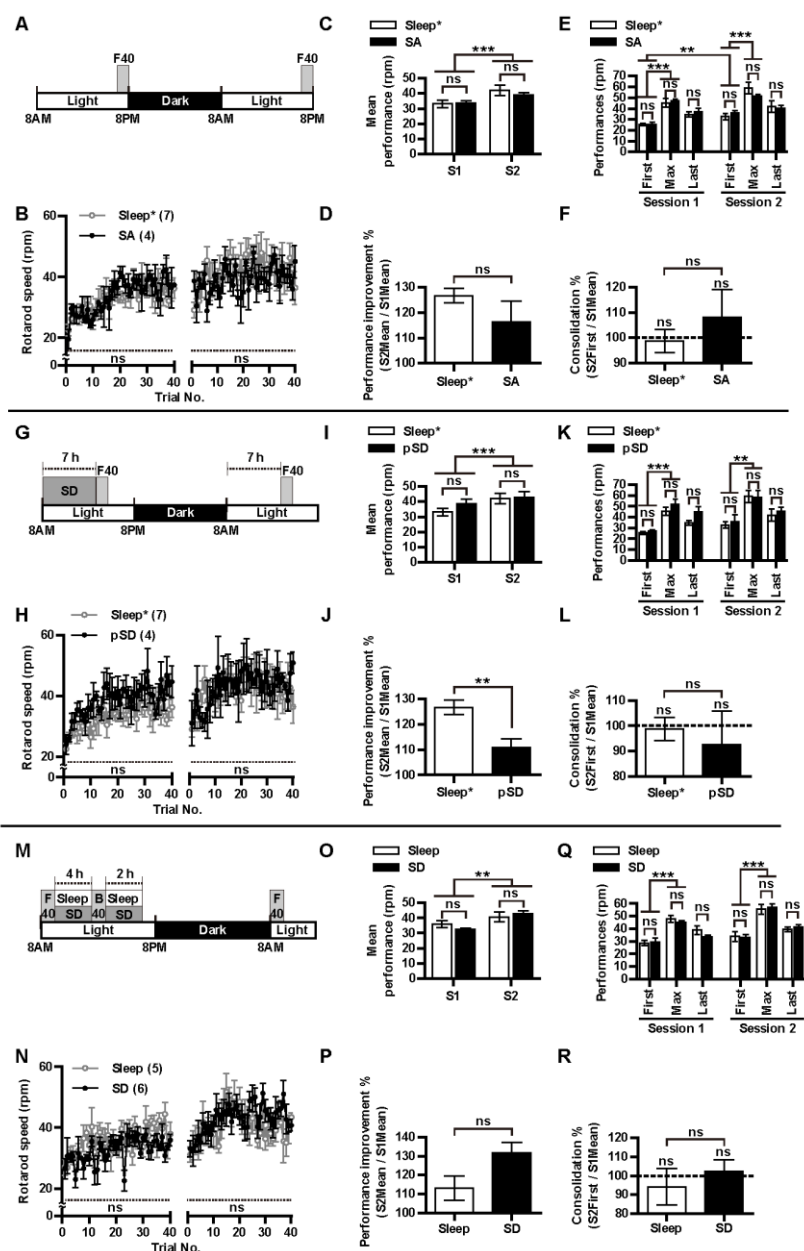
701 **Figure S1. No evidence for sleep-dependent consolidation in the rotarod task using 20 trials, different SD methods,**
702 **or when training is preceded by surgery.** (A-F) Experiment using two methods of SD and short training sessions (20
703 trials; 7 Sleep, 5 SDgh, 5 SDob). (G-L) Experiment using two methods of SD, short training sessions (20 trials) with a slow
704 acceleration profile, and with the second session immediately after 7h of sleep or SD (4 Sleep, 3 SDgh, 4 SDob). (M-R)
705 Mice received surgery and implantation of two EEG screws 24h prior to the first session of rotarod (40 trials /session; Sleep,
706 SDgh, 3 mice/group). Data are expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA followed by
707 Tukey post hoc test (D,F,J,L), Student's paired t test (within group comparison; F,L,R), Student's unpaired t test (P,R) and
708 two-way repeated measures ANOVA followed by Bonferroni post hoc test were used in the other panels.



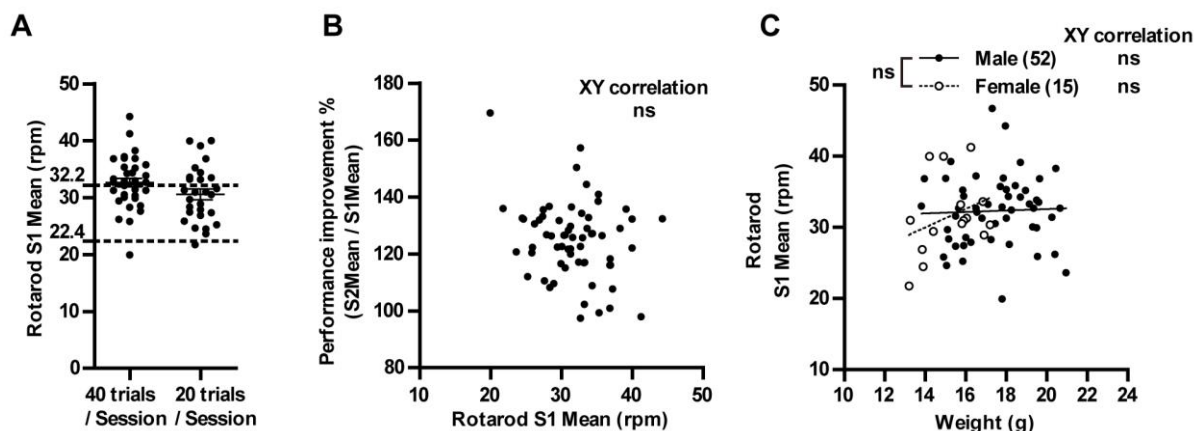
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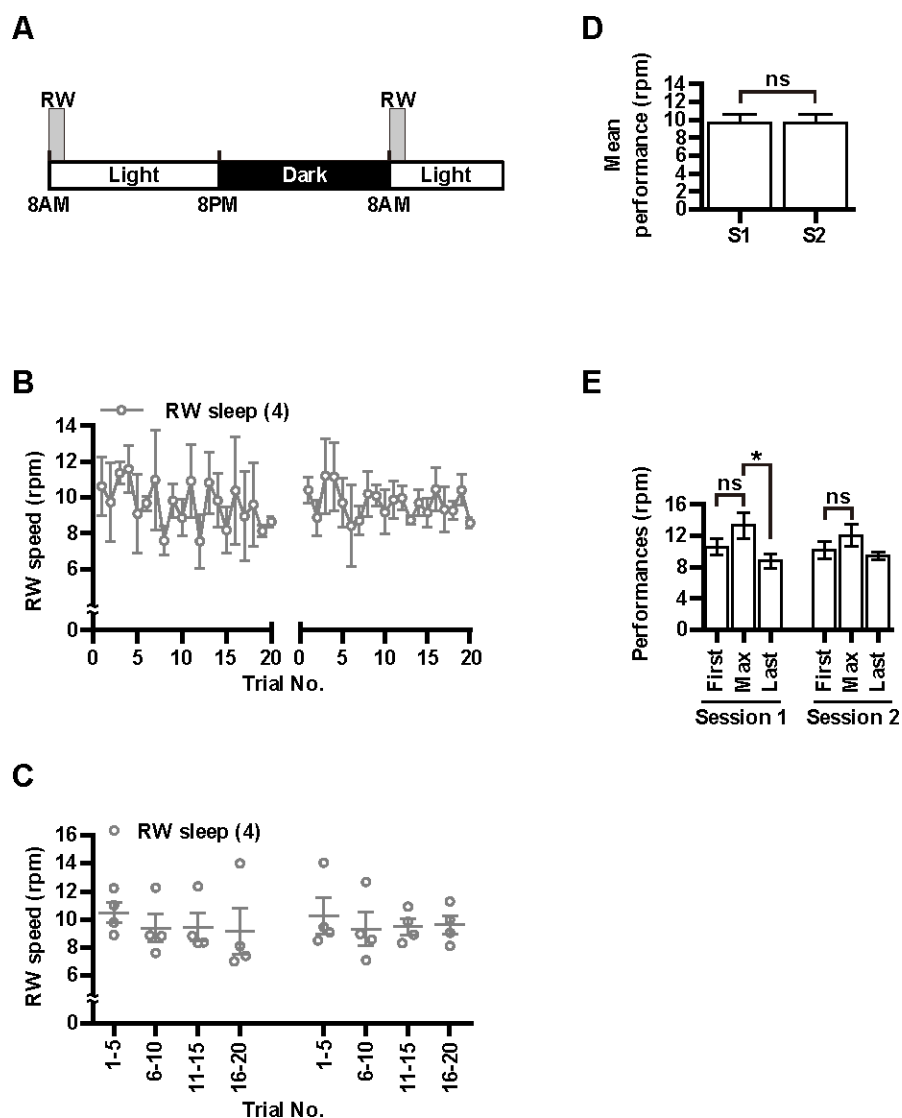
711 **Figure S2. No evidence for sleep-dependent consolidation as compared to spontaneous wake, and when training is**
712 **associated with interference. No effects of sleep on rotarod learning.** (A-F) Four mice received the first session of
713 rotarod training (40 trials) at the end of the light phase, followed by spontaneous wake during the dark period. *Sleep mice
714 are the same as in Fig.1. (G-L) Four mice were sleep deprived prior to the first session of rotarod training (40 trials) and
715 received the second session 24h after S1. * Sleep mice are the same as in Fig.1. (M-R) Mice received backward training (B,
716 40 trials) 4h after the first forward running session (F, 40 trials). SD occurred for 4h after F and for 2h after B. All mice (5
717 Sleep, 6 SD) were subjected to the second F session (40 trials) the next day. Data are expressed as mean \pm SEM. * $p < 0.05$,
718 ** $p < 0.01$, *** $p < 0.001$; Student's unpaired t test (D,F,J,L,P,R), Student's paired t test (within group comparison; F,L,R)
719 and two-way repeated measures ANOVA followed by Bonferroni post hoc test were used in the other panels.



721 **Figure S3. Overall analysis of rotarod learning.** (A) Individual data of S1 Mean in each mouse shown in Fig. 2A and B.
722 Dashed lines (32.2 and 22.4 rpm) indicate estimate of mean performance for sleep (32.2) and SD (22.4) mice in ²². (B)
723 Relationship between S1 Mean and performance improvement across sessions for each mouse shown in A and B. (C) Lack
724 of correlation between weight and S1 Mean performance (sex also did not correlate with performance). Values are
725 expressed as mean \pm SEM.; linear regression analysis, analysis of covariance and Spearman rank correlation test were used.

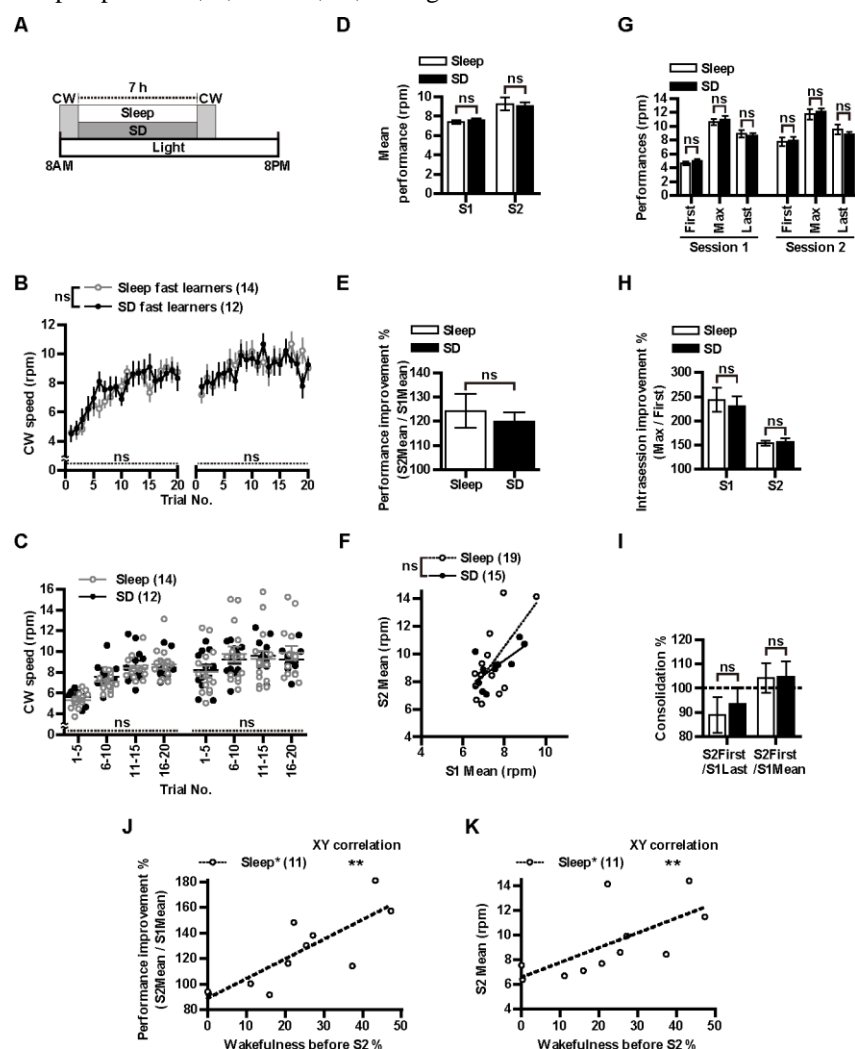


728 **Figure S4. Performance in a regular wheel.** (A) Schematic of the experiment. Mice were subjected to the first session (S1,
729 20 trials) of regular wheel task at 8AM and left undisturbed until the second session (S2, 20 trials) the next day. (B,C)
730 Performance shown for each single trial (B) and in bins of 5 trials (C). (D) Mean performance for each session. (E)
731 Performance measures for each session. Values are mean \pm SEM. * $p < 0.05$; Student's t test in (D) and one-way repeated
732 measures ANOVA followed by Tukey post hoc test was used in (E). RW, regular wheel; S, session; ns, not significant.
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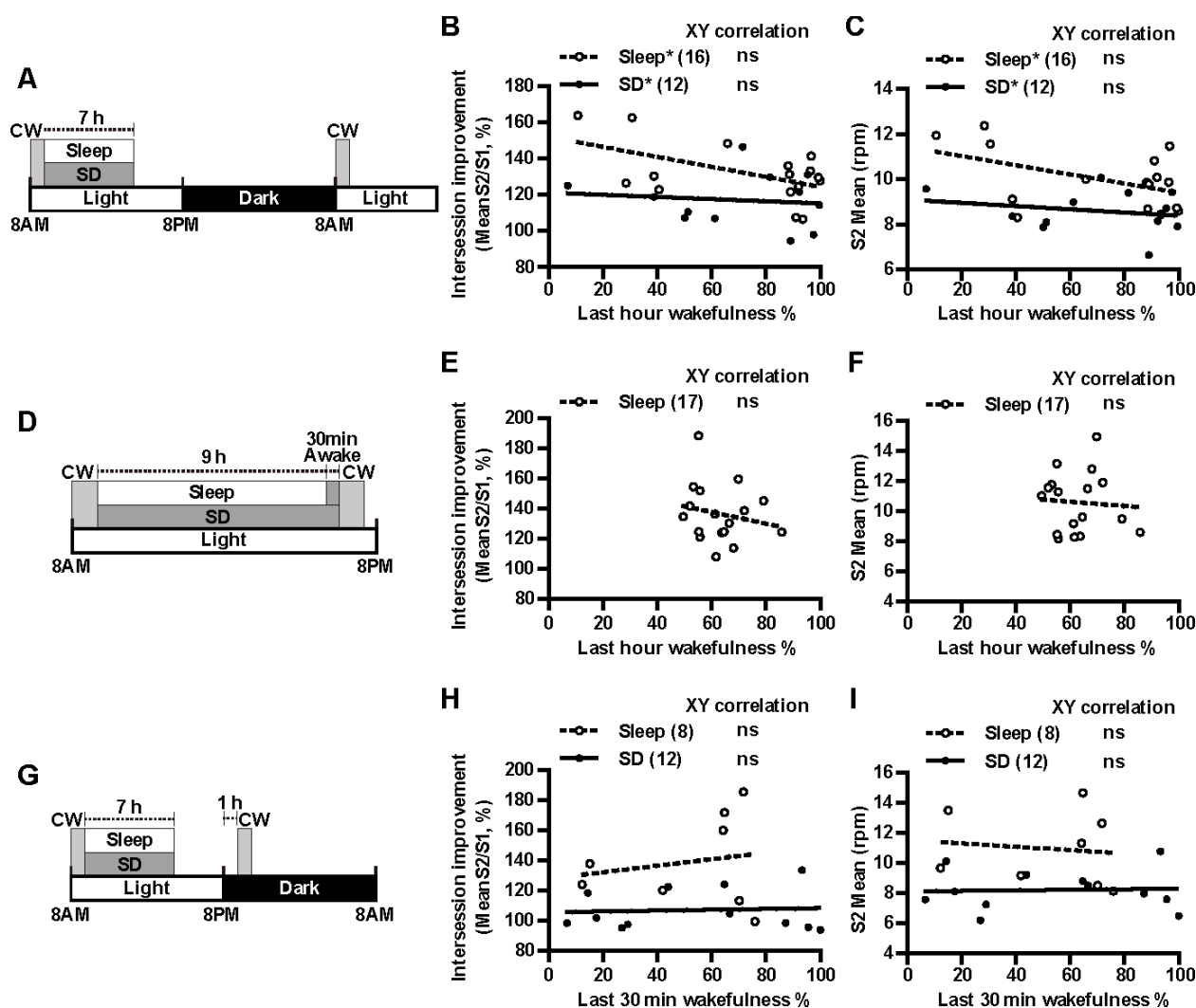


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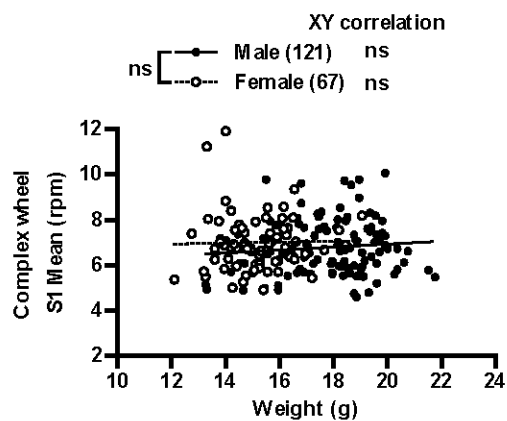
738 **Figure S5. The complex wheel task in a morning-to-afternoon paradigm: evidence for sleep inertia.** (A) Schematic of
739 the experiment. Mice were subjected to the first session (S1, 20 trials) of complex wheel task at 8AM and then divided in 2
740 groups (22 S, 15 SD) depending on whether in the following 7 h they could sleep or were sleep deprived by gentle handling.
741 Immediately after 7 h, both groups were trained again (S2, 20 trials). Only fast learners are shown in (B-K). (B,C)
742 Performance in each single trial (B) and in bins of 5 trials (C). (D) Mean performance for each session. (E) Performance
743 improvement across sessions. (f) Relationship between S1 Mean and S2 Mean for each mouse. Statistical significance was
744 calculated by comparing the linear regression lines of sleep and SD. (G) Performance measures for each session in the 2
745 groups. (H) Relative intrasession improvement. (I) Offline consolidation of motor skills using two measures. (J,K) Positive
746 correlation between time spent awake during the last hour before S2 and performance improvement across sessions (J) or
747 Mean S2 performance (k). Activity data of 3 mice was missing in (J,K). Values are mean \pm SEM. ** $p < 0.01$, *** $p < 0.001$;
748 two-way repeated measures ANOVA followed by either Bonferroni post hoc test or Student's t test was used in (B-D,G,H),
749 Student's t test in (E,I) and linear regression analysis followed by analysis of covariance and in (F,J,K). Correlations were
750 calculated using Spearman test (J) and Pearson test (K) based on the normality of distribution. CW, complex wheel; SD,
751 sleep deprivation; S, session; ns, not significant.



753 **Figure S6. No evidence for sleep inertia effects in the morning-to-morning, to-late afternoon and to-night paradigms**
754 **(fast learners).** (A-C) Morning-to-morning paradigm. Schematic of the experiment (A). No correlation between time spent
755 awake during the last hour before S2 and performance improvement across sessions (B) or S2 Mean (C). Activity data of 3
756 mice in each group was missing. (D-F) Morning-to-late afternoon paradigm. Schematic of the experiment (D). No
757 correlation between time spent awake during the last hour before S2 and performance improvement across sessions (E) or
758 S2 Mean (F) in sleep mice. The last hour before S2 includes 30 min exposure to novel objects. Since SD mice were almost
759 always awake before S2, their data are not shown. (G-I) Morning-to-night paradigm. Schematic of the experiment (G). No
760 correlation between time spent awake during the last 30 min before S2 and performance improvement across sessions (H)
761 or S2 Mean (I). * $p < 0.05$; Spearman test was used. CW, complex wheel; SD, sleep deprivation; S, session; ns, not
762 significant.
763



768 **Figure S7. Weight and sex do not correlate with motor performance in the complex wheel task.** Data of all fast
 769 learners and slow learners are shown. Linear regression analysis followed by analysis of covariance and Spearman test were
 770 used. CW, complex wheel; SD, sleep deprivation; S, session; ns, not significant.



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774 **Table S1. Summary data for all the mice used in the present study.**

775 Values are mean \pm SEM. IHC, immunohistochemistry; SD, sleep deprivation; ND, not determined.

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	Condition	Sex	No.	Age (day)	Weight (g)
Rotarod	Sleep	M	24	29.8 ± 0.1	17.2 ± 0.4
		F	2	29.5 ± 0.5	16 ± 0.3
	SD	M	28	30.2 ± 0.2	17.7 ± 0.3
		F	13	31 ± 0.3	15.1 ± 0.4
Complex wheel	Sleep	M	67	30.7 ± 0.2	17.7 ± 0.3
		F	37	31 ± 0.3	15.1 ± 0.2
	SD	M	54	30.5 ± 0.2	17.6 ± 0.2
		F	30	31.2 ± 0.4	15.3 ± 0.3
Regular wheel		M	3	30.0 ± 0.0	ND
		F	1	29	14.8
IHC - Fos		M	15	30.7 ± 0.3	16.9 ± 0.3

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783 **Table S2. Summary of all rotarod experiments.**

784 Values are mean \pm SEM. IHC, immunohistochemistry; S, sleep; SD, sleep deprivation; ND, not done; SA, spontaneously
785 awake; pSD, prior sleep deprivation

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Rotarod experiment	Timing of Session 2	Rod speed	Trial No.	Intervention	No. of mice		Significant Difference (S vs SD)
					Sleep	SD	
Yang et al.	Next day	Fast	40	Surgery	7	5	Yes
Fig.1	Next day	Fast	40	ND	7	7	No
Fig.S1A-F	Next day	Fast	20	ND	7	10	No
Fig.S1G-L	Immediately after S/SD	Slow	20	ND	4	7	No
Fig.S1M-R	Next day	Fast	40	Surgery	3	3	No
Fig.S2A-F	Next day	Fast	40	ND		4 (SA)	No
Fig.S2G-L	Next day	Fast	40	ND		4 (pSD)	No
Fig.S2M-R	Next day	Fast	40	Backward running	5	6	No

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791 **Movie S1. Training in the complex wheel task.** Note that the mouse comes back to the top of the wheel spontaneously,
792 suggesting that this task is not stressful.

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